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Practical Approach for Typing Strains of *Leishmania infantum* by Microsatellite Analysis

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Currently the universally accepted standard procedure for characterizing and identifying strains of *Leishmania* is isoenzyme analysis. However, in the Mediterranean area, despite their very wide geographical distribution, most *Leishmania infantum* strains belong to zymodeme MON-1. In order to increase our understanding of polymorphism in strains of *L. infantum*, we developed PCR assays amplifying 10 microsatellites and sequenced PCR products. The discriminative power of microsatellite analysis was tested by using a panel of 50 *L. infantum* strains collected from patients and dogs from Spain, France, and Israel, including 32 strains belonging to zymodeme MON-1, 8 strains belonging to zymodemes MON-24, MON-29, MON-33, MON-34, or MON-80, and 10 untyped strains. Five of the microsatellites were polymorphic, revealing 22 genotypes, whereas the five remaining microsatellites were not variable. In particular, MON-1 strains could be separated into 13 different closely related genotypes. MON-33 and MON-34 strains also gave two additional genotypes closely related to MON-1, while MON-29, MON-24, and MON 80 strains exhibited more divergent genotypes. Among the foci examined, the Catalonian focus displayed a high polymorphism, probably reflecting isoenzyme polymorphism, while the Israeli focus exhibited a low polymorphism that could be consistent with the recent reemergence and rapid spread of canine leishmaniasis in northern and central Israel. The strains originating from the south of France and the Madrid, Spain, area displayed significant microsatellite polymorphism even though they were monomorphic by isoenzyme analysis. In conclusion, microsatellite polymorphism exhibits a high discriminative power and appears to be suitable for characterization of closely related strains of *L. infantum* in epidemiological studies.

Protozoan parasites of the genus *Leishmania* cause a spectrum of diseases, ranging from self-limiting, self-curing cutaneous leishmaniasis (CL) to disseminating, fatal visceral leishmaniasis, and they infect various mammalian hosts. *Leishmania infantum* may cause either simple CL (18), debilitating visceral leishmaniasis, or asymptomatic cases. Currently, the universally accepted standard procedure for characterizing and identifying strains of *Leishmania* is isoenzyme analysis (3, 19, 22). However, this is performed only in a few laboratories and is, depending on the number of enzymes examined, very labor intensive and time consuming. Unlike some species of *Leishmania*, e.g., *Leishmania tropica* and *Leishmania major*, which exhibit extensive enzymatic polymorphism (20), most *L. infantum* strains isolated in Mediterranean foci belong to the sole predominant zymodeme MON-1, despite their very wide geographical distribution. Indeed, except in some foci, where significant enzymatic polymorphism has been found among strains of *L. infantum* (5, 16), in most Mediterranean foci, such as Provence in southern France or Israel, nearly all of the strains of *L. infantum* belong to the zymodeme MON-1 (11). In these foci, the insufficient discriminative power of isoenzyme typing methods prevents researchers from establishing correlations between clinical feature, preferential host (dogs, immunocompetent children, and human immunodeficiency virus [HIV]-infected patients), and particular group of strains. In the same way, the lack of discrimination between strains also prevents genetic studies on parasite populations.

Several molecular biological typing methods have been developed to improve the discriminative power of typing methods for the genus *Leishmania*. These include amplification of a parasite DNA sequence by either a specific PCR or a random amplified polymorphic DNA (RAPD) PCR or detection of restriction fragment length polymorphisms (RFLPs) by Southern hybridization with DNA-specific probes (11, 17). The last two methods have drawbacks. RFLP analysis is a time-consuming technique and large amounts of purified DNA are needed (2), whereas RAPD analysis requires strict conditions to obtain reproducibility between different laboratories and generates complex patterns (13, 23). In contrast, specific PCR-based methods are attractive because of their rapidity and because culturing parasites can be avoided (9). However, in most cases, the level of polymorphism found with coding or repeated noncoding PCR-amplified sequences is not refined enough to dis-

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tinguish between closely related strains (6, 8, 24). Microsatellite DNA sequences, tandem repeats of a simple nucleotide motif, are distributed abundantly in the eukaryotic genomes and may reveal important strain polymorphisms. However, until now only two microsatellites showing size polymorphism have been identified and characterized for *L. infantum*. Usually, to study microsatellites, investigators screen a genomic DNA library and then evaluate the microsatellite size polymorphism by PCR amplification and electrophoresis on acrylamide gels (21). In the present study, we looked for new microsatellites in the genome of *Leishmania* without performing genomic library screening. Microsatellites were selected from leishmanial DNA sequences published in data banks and particularly from the *L. major* chromosome 1 genome. The polymorphism of the microsatellite DNA sequences was evaluated by comparative analysis of the PCR product sequences of 50 strains of *L. infantum* collected in four Mediterranean regions of endemicity: Catalonia and Madrid, Spain; Provence, France; and northern and central Israel.

MATERIALS AND METHODS

Strategy. Potential targets for PCR amplification were selected from the leishmanial DNA sequences available in the GenBank database. Since most of them were located on chromosome 1 of *L. major* MHOM/IL/81/Friedlin (12), we used a pragmatic strategy to obtain the corresponding sequences from the genome of *L. infantum*. First, we selected, among sequences possessing microsatellites, those of less than 1 kb presenting coding sequences at both extremities. Second, we selected primers that recognized the coding part of the sequences and performed PCR, with DNA from *L. infantum* as the template. Third, we sequenced PCR products to locate the microsatellites and design new primers closer to these regions. Finally, we selected three DNA targets in the genome of *L. infantum*. Two of these targets corresponded to sequences originally found in the genome of *L. major*, one which contains three microsatellites (the Lm2 sequence) and another which contains four microsatellites (the Lm4 sequence). The third target was the internal transcribed spacer (ITS) region, which contains three additional microsatellites.

Strains. Fifty strains were used in this study (Table 1). They were isolated from 48 hosts in three Mediterranean countries: Spain, France, and Israel. Forty of them had been characterized and identified previously as *L. infantum* by isoenzyme analysis. In two cases, two strains were isolated from separate samples collected from the same human host (BCN 143/BCN 167 and BCN 224/BCN 226) during successive episodes of leishmaniasis. Fourteen strains were collected in Provence, France. This region represents a homogeneous focus in terms of leishmanial parasites because all the strains collected from it so far have belonged to the zymodeme MON-1, including those isolated from patients coinfected with HIV. Twenty strains were isolated in Catalonia, Spain, from dogs and both immunocompromised and immunocompetent patients. The Catalonian focus is more heterogeneous in its leishmanial parasites and includes strains belonging to the predominant zymodeme MON-1 and other zymodemes (MON-24, MON-29, MON-33, MON-34, and MON-80). Additional Spanish strains, all belonging to MON-1, were collected in Madrid from dogs. Ten strains came from central and northern Israel. Canine leishmaniasis has recently reemerged in central Israel after an apparent absence of more than 40 years (1). All 10 strains were, essentially, isolated from dogs. One of them, strain LRC-L760*, was isolated from a colony-bred sandfly, a South American *Lutzomyia longipalpis*, that had fed on a local dog undergoing treatment with allopurinol during xenodiagnosis. Its sibling strain, LRC-L760, isolated from the same dog before treatment directly into rabbit blood agar semisolid medium was identified as *L. infantum* zymodeme MON-1 by isoenzyme analysis. The other nine Israeli strains were not characterized by isoenzyme analysis. However, 13 other Israeli strains of *L. infantum* from dogs and humans sent to Montpellier, France, for isoenzyme analysis were all zymodeme MON-1.

In addition, the primers used in this study were tested on one strain of *L. major* (LPM 193) (data not shown). This strain belongs to zymodeme MON-26 and was isolated in 1999 from a patient from Saudi Arabia with CL.

Isolation and growth of parasites. The strains were isolated and grown as promastigotes on Novy-MacNeal-Nicolle biphasic culture medium.

TABLE 1. Main features of strains of *L. infantum* collected in three Mediterranean foci

International code	Zymodeme	Host	HIV status	Origin
MHOM/ES/84/BCN 1	MON-29	Human	−	Barcelona, Spain
MCAN/ES/92/BCN 83	MON-1	Dog	−	Tarragona, Spain
MHOM/ES/94/BCN 143	MON-33	Human	+	Barcelona, Spain
MHOM/ES/96/BCN 167	MON-33	Human	+	Barcelona, Spain
MHOM/ES/97/BCN 186	MON-34	Human	+	Barcelona, Spain
MHOM/ES/99/BCN 214	MON-1	Human	−	Barcelona, Spain
MHOM/ES/99/BCN 224	MON-24	Human	+	Barcelona, Spain
MHOM/ES/99/BCN 225	MON-1	Human	+	Barcelona, Spain
MHOM/ES/99/BCN 226	MON-24	Human	+	Barcelona, Spain
MCAN/ES/99/BCN 234	MON-1	Dog		Tarragona, Spain
MCAN/ES/99/BCN 235	MON-1	Dog		Tarragona, Spain
MCAN/ES/99/BCN 236	MON-1	Dog		Tarragona, Spain
MCAN/ES/99/BCN 237	MON-1	Dog		Tarragona, Spain
MCAN/ES/99/BCN 241	MON-1	Dog		Tarragona, Spain
MCAN/ES/99/BCN 242	MON-1	Dog		Tarragona, Spain
MHOM/ES/00/BCN 258	MON-80	Human	−	Barcelona, Spain
MHOM/ES/00/BCN 259	MON-80	Human	+	Barcelona, Spain
MCAN/ES/00/BCN 264	NA[a]	Dog		Tarragona, Spain
MCAN/ES/00/BCN 265	MON-1	Dog		Tarragona, Spain
MCAN/ES/00/BCN 266	MON-1	Dog		Tarragona, Spain
MCAN/ES/00/UCM1	MON-1	Dog		Madrid, Spain
MCAN/ES/00/UCM3	MON-1	Dog		Madrid, Spain
MCAN/ES/00/UCM4	MON-1	Dog		Madrid, Spain
MCAN/ES/00/UCM6	MON-1	Dog		Madrid, Spain
MCAN/ES/00/UCM10	MON-1	Dog		Madrid, Spain
MCAN/ES/00UCM11	MON-1	Dog		Madrid, Spain
MHOM/FR/99/LPM 190	MON-1	Human	−	Provence, France
MHOM/FR/99/LPM 191	MON-1	Human	+	Provence, France
MHOM/FR/99/LPM 192	MON-1	Human	−	Provence, France
MHOM/FR/99/LPM 194	MON-1	Human	−	Provence, France
MHOM/FR/99/LPM 195	MON-1	Human	+	Provence, France
MHOM/FR/00/LPM 196	MON-1	Human	+	Provence, France
MHOM/FR/00/LPM 197	MON-1	Human	+	Provence, France
MHOM/FR/00/LPM 199	MON-1	Human	−	Provence, France
MHOM/FR/00/LPM 200	MON-1	Human	+	Provence, France
MHOM/FR/00/LPM 201	MON-1	Human	+	Provence, France
MHOM/FR/00/LPM 202	MON-1	Human	−	Provence, France
MHOM/FR/00/LPM 203	NA	Human	−	Provence, France
MHOM/FR/00/LPM 204	MON-1	Human	−	Provence, France
MHOM/FR/00/LPM 205	MON-1	Human	−	Provence, France
MCAN/IL/98/LRC-L741	MON-1	Dog		Central Israel
MCAN/IL/99/LRC-L760	MON-1[b]	Dog		Central Israel
MCAN/IL/99/LRC-L760*		Dog via sandfly		
MCAN/IL/00/LRC-L787	NA	Dog		Central Israel
MCAN/IL/00/LRC-L789	NA	Dog		Central Israel
MCAN/IL/00/LRC-L792	NA	Dog		Central Israel
MCAN/IL/00/LRC-L798	NA	Dog		Northern Israel
MCAN/IL/00/LRC-L799	NA	Dog		Central Israel
MCAN/IL/00/LRC-L800	NA	Dog		Central Israel
MCAN/IL/00/LRC-L801	NA	Dog		Northern Israel
MCAN/IL/00/LRC-L802	NA	Dog		Northern Israel

[a] NA, not available.
[b] Refer to "Strains" in Materials and Methods.

DNA preparation. The total DNA was extracted from the promastigotes for PCR amplification. Samples of 100 μl of culture were washed in 0.3% NaCl. The pellet was lysed by heating at 96°C for 20 min with 400 μl of a mixture containing 1% Tween 20 (Sigma, St. Louis, Mo.), 1% Nonidet P-40 (Sigma), and 20% Chelex resin (Bio-Rad, Hercules, Calif.) made up in sterile distilled water. The mixture was then centrifuged at 14,000 × *g* for 10 min at room temperature. The supernatant was collected and was either used immediately for PCR amplification or stored at −20°C until used.

PCR amplification. Three PCRs were performed. The primers A2 (5′-GGG AGAAGCTCTATTGTG-3′) and B1 (5′-ACACTCAGGTCTGTAAAC-3′) were used for analyzing the ITS region, and primers Lm4A1 (5′-CGGTGCAC ATTCGACCGCTA-3′) and Lm4B1 (5′-ATGGCACGGTGCACGCTTCC-3′) were used for amplifying the Lm4 sequence. Two pairs of primers were used to

amplify the Lm2 sequence. First, we amplified the Lm2 sequence with Lm2A1 (5′-TGACGCGACGTGGCAAGTCA-3′) and Lm2B1 (5′-CCGTGAAGTACT CGGACGCT-3′), primers that recognize a 900-bp-long fragment. Then we used internal primers, Lm2A3 (5′-AAAAAGCGAGGAATGAAAGAA-3′) and Lm2B3 (5′-TAGAGGCGTGGCAGAGAC-3′), which were localized on either side of the most variable microsatellite, for sequencing the complete Lm2 fragment in both directions. All the primers were synthesized by Eurogentec (Seraing, Belgium).

Each reaction mixture (50 μl) contained a 0.05 mM concentration of each deoxynucleoside triphosphate, a 0.5 μM concentration of each primer, 1× REDtaq buffer, 1 U of REDtaq polymerase (Sigma), and 5 μl of total DNA extract. After a 2-min incubation at 94°C, amplification of either the ITS region or the Lm2 and Lm4 sequences was performed for 40 or 35 cycles, respectively, in an automated thermocycler PTJ 100 (MJ Research, Inc, Watertown, Mass.). The conditions used for the ITS region were denaturation at 94°C for 20 s, primer annealing at 53°C for 30 s, and extension at 72°C for 1 min. The conditions used for the other sequences were denaturation for 30 s at 94°C, primer annealing for 30 s at 63°C, and extension for 1 min at 72°C for the Lm4 sequence; denaturation for 30 s at 94°C, primer annealing for 30 s at 62°C, and extension for 90 s at 72°C for the Lm2 sequence with the Lm2A1 and Lm2B1 primers; and denaturation for 30 s at 94°C, primer annealing for 30 s at 51°C, and extension for 1 min at 72°C for the Lm2 sequence with the Lm2A3 and Lm2B3 primers.

After amplification, 10 μl of PCR products was visualized by electrophoresis on a 1.5% agarose gel containing ethidium bromide.

DNA sequencing. Amplified DNA was purified by using the QIAquick PCR purification kit (Qiagen SA, Courtaboeuf, France) according to the manufacturer's recommendations. The purified DNA for each strain was then sequenced in both directions with a fluorescent labeling kit (Thermo Sequenase II kit; Amersham Pharmacia Biotech Europe, Orsay, France) with specific primers on an automated DNA sequencer 373A (Perkin Elmer Applied Biosystems, Foster City, Calif.).

In some cases, the results obtained by direct sequencing of Lm2 PCR products were confirmed after cloning the PCR products into the vector pCR 2.1 (TA Cloning kit; Invitrogen, Leek, The Netherlands).

Phenetic dendrogram. The ITS, Lm2, and Lm4 sequences obtained for each different genotype were aligned, and a dendrogram was calculated with the fastDNAml software (version 1.2.2) (4, 14). TREEVIEW (15), a tree drawing software, was used to produce the tree.

Reproducibility of PCR and stability of the genotypes. Reproducibility was assessed by repeating the preparation of the DNA from the two strains UCM 1 and LPM 191 six times followed by amplification and sequencing of all of the samples. In addition, the primers A2 and B1 were used repetitively, six times, to amplify the ITS sequence from three DNA extracts of the strains BCN 1, BCN 143, and BCN 226. Similarly, six amplifications were performed with DNA isolated from the five strains BCN 1, BCN 143, BCN 235, BCN 258, and LPM 191 with the primers Lm4A1 and Lm4B1.

We also verified the stability of the strains in vitro and in vivo. After culturing the promastigotes of strains BCN 1, BCN 224, and LPM 195 in six different batches of Novy-MacNeal-Nicolle medium over 3 months, their DNAs were isolated and PCRs were performed for the three molecular targets. In parallel, total DNA was extracted at each stage from the leishmanial promastigotes of strain BCN 83 grown directly after isolation from a dog, regrown after passage through and reisolation from a hamster, and then regrown after passage through and reisolation from another dog. All of these DNA extracts were submitted to PCR amplification and sequencing.

RESULTS

Sequence analysis of the PCR-amplified ITS region with primers A2 and B1. All strains gave an amplified fragment of about 650 bp. The A2B1 part of the ITS sequence contained three microsatellites exhibiting a moderate degree of size polymorphism: the number of AT repeat units was 5 or 6, the cluster TA was repeated 4 or 5 times, and the number of G repeat units was 5, 7, or 8 (Fig. 1A).

Three different sequences were found among the strains from Catalonia, whereas all strains from Madrid, Provence, and Israel had the same sequence (Table 2).

Sequence analysis of the Lm2 and Lm4 fragments. Amplification of the Lm2 region generated an amplified fragment of about 900 bp for *L. infantum* and one of about 750 bp for *L. major*. The Lm2 sequence exhibited four microsatellites. Three of them corresponding to a C_7 microsatellite, a $(CA)_8$ microsatellite, and an imperfect $(TG)_8$-CG-$(TG)_5$ microsatellite were identical among all strains. The last one, corresponding to a poly-TG, was highly variable; the dinucleotide was repeated 17, 22, 23, 24, 25, 26, or 27 times (Fig. 1B). No differences were found between sequences obtained directly after PCR or after cloning of the PCR products.

Finally, sequence analysis of the Lm2 PCR products in all strains identified seven profiles only due to the size variation of the poly-TG (Table 2). Six of them were found for the strains originating from Catalonia. Three different Lm2 sequences were identified among strains collected in Provence and Israel.

Amplification with primers Lm4A1 and Lm4B1 produced a fragment of 560 bp for each species, *L. infantum* and *L. major*. The Lm4 region contained three microsatellites: poly-A, poly-T, and poly-TA. Two of them were identical among all strains (A_{10} and T_9), while the $(TA)_n$ microsatellite exhibited a high level of size polymorphism. The TA unit was repeated 9, 10, 11, 12, 13, or 14 times (Fig. 1C).

Finally, sequence analysis of the Lm4 PCR products in all strains identified six profiles due only to the size variation of the poly-TA (Table 2). Strains from Israel had the same Lm4 sequence while strains from Madrid, Catalonia, and Provence gave three, four, and five different sequences, respectively.

Reproducibility of PCR and stability of the genotypes. The ITS, Lm2, and Lm4 sequences obtained for each strain were identical when six different preparations of the same DNA were used for the PCRs or when the fragment was amplified six times by using the same DNA. In addition, the ITS, Lm2, and Lm4 sequences showed no variations even after multiple generations in vivo and in vitro.

Results comparison. The ITS-, Lm2-, and Lm4-associated sequences generated 22 genotypes for 50 strains of *L. infantum* belonging to six zymodemes (Table 2). In particular, the MON-1 strains gave thirteen different genotypes that were closely related to each other (Fig. 2). In addition, DNA typing of strains MON-33 and MON-34 gave two different genotypes that were closely related to the MON-1 genotype. On the other hand, strains MON-29, MON-24, and MON-80 showed more-divergent genotypes.

Even when more than one strain was isolated from the same patient, the strains still had identical genotypes. Strains collected in the Provence region, which all belong to the zymodeme MON-1, gave nine different genotypes, while the 10 *L. infantum* strains originating from northern and central Israel resulted in only three genotypes.

DISCUSSION

We have developed a reproducible, discriminating molecular typing system that was able to characterize and distinguish closely related strains of *L. infantum* collected in three Mediterranean countries: France, Spain, and Israel. It is based on the comparative analysis of PCR products from three nuclear noncoding DNA sequences containing polymorphic microsat-

A

AGTGTCGAACAAAAAACAACACGCCGCCTCCTCTCTTCTGCAC**(AT)**$^{5-6}$TATACCATACA
CAG**(TA)**$^{4-5}$ATTATGTGTTGGAAGCCAAGAGGAGGCGTGTGTTTGTGTTGTGCGCATATTATAT
GTATATATGCTGTGTGCACACGTAGACAAGTTAGAGTTGGACAAATACACACATGCACTCTCTT
TTGTGTGGGTGCGCGCGTGGAAACTCCTCTCTGGTGCTTGCAAAGCAGTCTTTTTCTCTTTCTC
TTTTTCTCTCTCCATTCTCTCCTCTCTTTTTTCATCAAAAAGGGGGGAGAGAAAAAGAGAGAGG
A**(G)**$^{5-8}$TCGAGGGAGAGAGGCTGTGACCAGGATTATTAAACAAAAAACCAAAACGAGAATTCAA
CTTCGCGTTGGCCATTTTTTGCTTAATGGGGGGAGGTGGGTGTGGGTGGTGTGTGGCTCTCTCT
CTGTGTGGTATATATATATGTATATTAGAAGTAGGTTGTGTGTGTGTGTATGTGTTTTACACAT
ATATATATCCGCGCCCTCACTCTCTCATATATAATTTATA

B

AAGGAGAGGGTACCCCGCTTCCGCCCG**(C)**^{7}TCCTCTTCCTCGCTAACCACGGACGCAC
CGCCCACACATCCAGGCAGTGGTGTGCACCGGAGTACTCTTCCTGCTCATGCACGTGTGTGTG
TGCGTGTGCGTGCACGGCCAGTCCTGCCAGCACGTCAACTGCTGCTGCTAAACTTGTGACGGA
CGCCACCACCTCCGCTCTCCCGACGCTTTCTGTTGTACGCCACATCGACGTGAAGGGCAACAC
GAAAAAAGCGAGGAATGAAAGAAGTAGATGAGCACACGCGCGCGCACACACAGGTGTGCTCTG
CA**(TG)**$^{17-27}$GGCGTGTGGAAGGTTGTAGAGGGGAGGGAGTGGGAGGGCGATGCGGCAGGCTG
CTCTGCTGCATGCAATCCACTGCGTCTGTGTACACTTGTAGGCACGTGTGGCGGTGCGACGAC
GCTGTGCACTCACTGTAGTCAGCTGGCACACTTGTGATCTGGCGTACGGTGA**(TG)**^{8}**CG(TG)**
^{5}GCGTGTTGCTCTGCTCTTCCCTTCTGCCTCAACCGCCACCACCTCAGCCACCATATTGTGCG
GTGACATCGTCTCTGCCACGCCTCTACTCGGCGCACATCTCTGCCTCTGCTGTCCCTCACAAA
CATTTGCTTCTTTACACATGCACGCACACGCACG**(CA)**^{8}TGCACACATGAACGCGTGCTGGAT
GATGGGGCGTTAACAGCTCCGCTCCATCGACGGGCAGAAGCGAGATTCAACAGCAAGCGAAAA
AGGAAACTGTGAAGCGGCGAGGGCAGACAATG

C

AATGTGGTCTCACTAGCTTTCAATTCACTGTCTGAATTTTTTCCGTTCTTCTGTGTATA
CGGTGCAGGCGGTTGATTGTGTTTCGTAACGCTTGGCATTTTCGACTCTGAGATTTCAGACCT
CCGTCGTCGAG**(A)**^{10}CTCTTTTCTTGCGTCGCTAGTGGCCCTCCTTGCCCCTCCAATCCGGG
GTGCACGCCGGAAAACAAGCCTCGTTCATGGCAGATAGCTCCCTATATTTCACGTCCTCGGAA
GTGTATCTCACTTCCCATTTTCTCCACATGATCTCTCTTCCCTCACGCTCTCTCATTACACGA
ATGTGTCATTTTTGAATTGGGAACC**(T)**^{9}GCCACACACATACACTTAGTAAAGGTG**(TA)**$^{9-14}$
TTTCGGTGCTCGCGATGTCGTCTACAGTGTACGAATTC

FIG. 1. Complete nucleotide sequences of genomic DNA regions containing microsatellites from strains of *L. infantum*. Microsatellites are shown in bold type. The superscripts indicate the minimum and maximum numbers of repeat units found for each microsatellite. (A) Nucleotide sequence of PCR product determined with primers A2 and B1. (B) Nucleotide sequence of PCR product determined first with primers Lm2A1 and Lm2B1 and then with primers Lm2A3 and Lm2B3. The internal primers, Lm2A3 and Lm2B3, are underlined. (C) Nucleotide sequence of PCR product determined with primers Lm4A1 and Lm4B1.

ellites. The partial screening of a leishmanial genomic library was not needed to identify these microsatellites. Instead, polymorphic targets were selected from among complete DNA sequences published in data banks (12). Since many such sequences are available in the gene data banks, this strategy can be applied to the molecular typing of other eukaryotic microorganisms.

One major advantage of this approach is its simplification of *Leishmania* strain typing in the Mediterranean region. By employing parasite-specific primers to polymorphic microsatellites, parasite culturing may be avoided in many cases. PCR could be carried out directly with infected host tissue, since nonspecific amplification of human or dog DNA with these primers was not observed (data not shown). In addition, this approach for typing strains of *Leishmania* is rapid. It takes just a few days to perform DNA extraction, amplification by PCR, and sequencing, whereas isoenzyme analysis, which is still the "gold standard," may take several months from strain isolation through culture to provide a full enzyme profile and zymodeme designation. The usefulness of isoenzyme analysis for epidemiological and taxonomic studies has been widely proven, but microsatellite analysis seems to be more discriminating with the 15 different genotypes found for MON-1 in our study. This genetic heterogeneity of strains belonging to one zymodeme has been observed for other approaches based on DNA microheterogeniety, such as RAPD and RFLP PCR methods (7, 10, 23).

The new typing approach presented here is very reproducible; repeat testing of the same isolate after multiple generations in vivo and in vitro gave identical sequences. However, in some cases, sequences of very long microsatellites, such as the poly-TG found in the Lm2 sequence, were quite difficult to read, and ambiguities were observed at the end of the microsatellite. These ambiguities could be resolved by sequencing the reverse DNA strand. Since sequences obtained after PCR cloning were strictly identical, we think that this problem does not reflect a sequence polymorphism within the population of *Leishmania* in the sample. This problem is more likely due to DNA polymerase misreading during PCR amplification.

Among the three sequences (ITS, Lm2, and Lm4) analyzed, only the microsatellite region was variable and size polymorphism was the only variation noted in the microsatellites. No

TABLE 2. Comparison of isoenzyme analysis and DNA typing by microsatellite size polymorphism with *L. infantum*

No. of repeats in:					Strain(s) from:				Zymodeme
ITS region			Lm2 (TG) sequence	Lm4 (TA) sequence					
AT	TA	G			Catalonia, Spain	Madrid, Spain	Provence, France	Israel	
5	5	7	22	10	BCN 143, BCN 167				MON-33
5	5	7	24	12			LPM 190, LPM 205		MON-1
5	5	7	24	13			LPM 192, LPM 204		MON-1
							LPM 203		NA[a]
5	5	7	24	14			LPM 191		MON-1
5	5	7	25	10			LPM 199		MON-1
5	5	7	25	11	BCN 214, BCN 241, BCN 242				MON-1
5	5	7	25	12	BCN 83, BCN 234, BCN 266		LPM 196		MON-1
5	5	7	25	13			LPM 200, LPM 201		MON-1
5	5	7	25	14			LPM 194, LPM 195	LRC-L741	MON-1
								LRC-L787, LRC-L789, LRC-L792, LRC-L799, LRC-L800	NA
5	5	7	26	9			LPM 202		MON-1
5	5	7	26	10	BCN 265	UCM 11	LPM 197		MON-1
5	5	7	26	12	BCN 225, BCN 237	UCM 1, UCM 3, UCM 4			MON-1
5	5	7	26	13		UCM 6			MON-1
5	5	7	26	14				LRC-L798, LRC-L801	NA
								LRC-L802	NA
5	5	7	27	11	BCN 235				MON-1
					BCN 264				NA
5	5	7	27	12	BCN 236				MON-1
5	5	7	27	14				LRC-L760*, LRC-L760	MON-1[b]
5	5	7	27	13	BCN 186				MON-34
						UCM 10			MON-1
5	5	8	23	13	BCN 1				MON-29
5	5	8	25	12	BCN 259				MON-80
5	5	8	26	12	BCN 258				MON-80
6	4	5	17	10	BCN 224, BCN 226				MON-24

[a] NA, not available.
[b] Refer to "Strains" in Materials and Methods.

exchanges of bases were seen in or around microsatellite clusters. This is probably due to the fact that all strains of the panel are closely related. As microsatellites exhibit only size variation and sequencing of very long microsatellites is sometimes difficult to perform, it should be possible to improve this technique further. Indeed, size polymorphism of microsatellites could be evaluated by acrylamide gel electrophoresis of fluorescein-labeled PCR products (2). This would save time and expense because DNA sequencing of the PCR products could be avoided. The microsatellites analyzed here displayed variable levels of polymorphism that may be related to the number of repeat units and/or the composition of the microsatellite. To verify this hypothesis, analysis of more microsatellites would clearly be useful. These data could be used to select more polymorphic microsatellites and increase the discriminatory power of such typing methods. In addition, it is important to note that our results are not in contradiction with the isoenzyme typing data (Fig. 2).

Between the different foci of leishmaniasis analyzed, the polymorphism observed with the five microsatellites varied. The Catalonian focus displayed the highest genomic polymorphism and probably reflects the isoenzyme polymorphism observed in that region. The Israeli focus displayed less genomic polymorphism among its strains, perhaps correlated with the recent reemergence and rapid spread of canine leishmaniasis caused by *L. infantum* in northern and central Israel (1). The strains originating from the south of France displayed significant microsatellite size polymorphism even though they were monomorphic by enzyme analysis. Concerning the Madrid focus, its relative stability (1 zymodeme and 4 closely related genotypes) seems to be correlated with the smaller area in which the strains were obtained.

To date, we have only studied strains of *L. infantum* isolated from humans and dogs. However, it should be possible to use microsatellite variation to study the genetic diversity of leishmanial parasites from all of the possible different situations, i.e., different clinical presentations, human (immunocompromised or immunocompetent children) and animal (dogs and wild canids) hosts, and sandfly vectors existing in the same area of endemicity. Microsatellites might also prove useful in determining whether or not there is any relationship between genotype and the pathogenicity of *Leishmania* strains. This technique could be useful in discerning whether specific leishmanial genotypes confer host specificity and if leishmanial parasite diversity is consistent in different hosts.

In conclusion, microsatellite polymorphism is simply and rapidly detected by PCR, displays a high level of reproducibility, and exhibits a high level of discrimination. It is suitable for characterizing closely related strains of *L. infantum*. Since

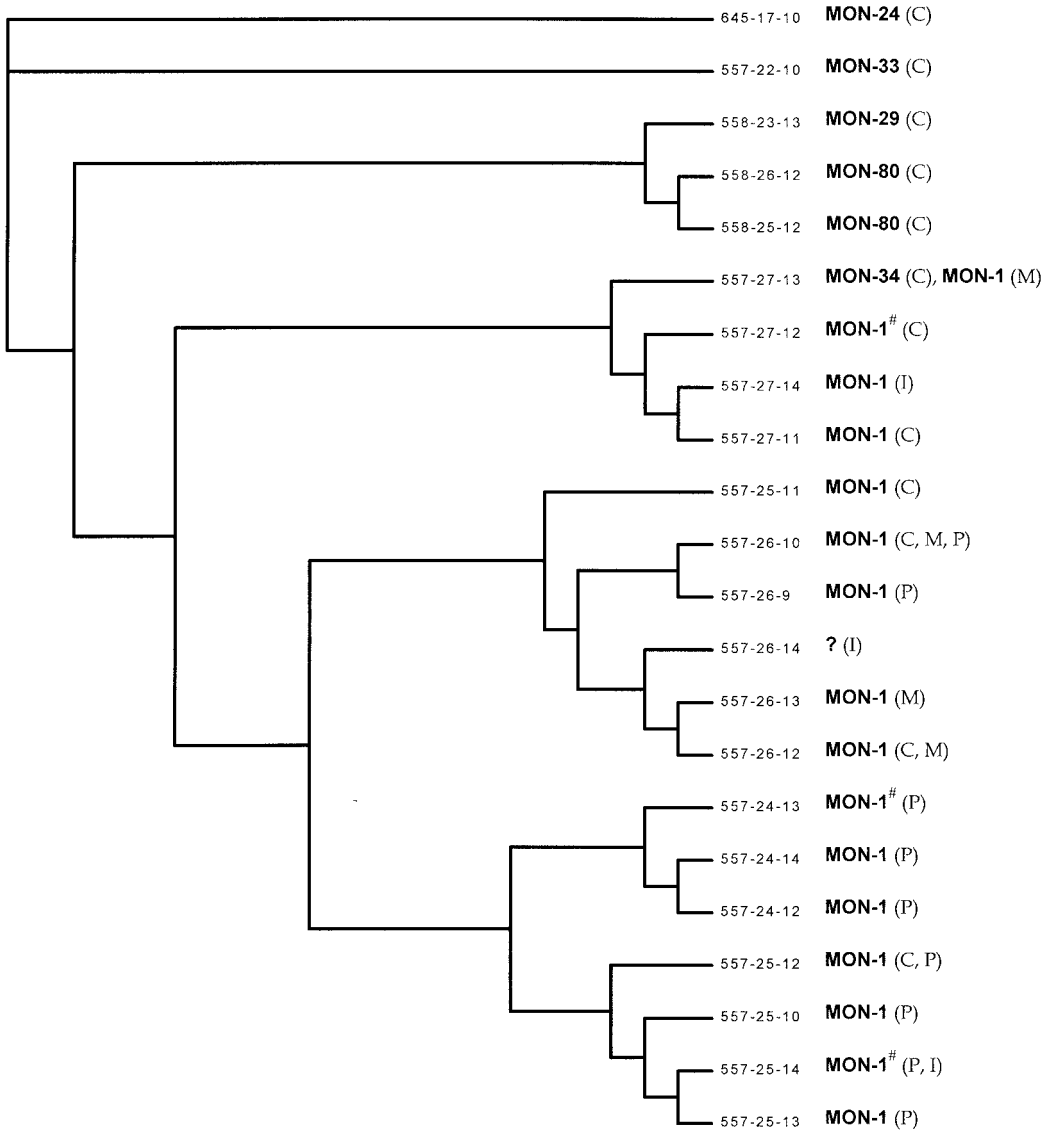

FIG. 2. Dendrogram calculated with fastDNAml (version 1.2.2), which is based in part on Felsenstein's nucleic acid sequence maximum likelihood method (version 3.3), and elaborated with TREEVIEW tree drawing software. Each genotype is characterized by its microsatellite combination (ITS-Lm2-Lm4), its zymodeme, and its geographical origin. C, Catalonia; M, Madrid; P, Provence; I, Israel; #, the zymodeme is not available for all of the samples of the genotype (Table 2).

many sequences are published in existing data banks, the strategy used here could be applied to the molecular typing of other eukaryotic microorganisms.

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